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ABSTRACT

A relatively sensitive quantitative method is described for recovering small quantities of virus from large quantities of liquid. Recent recovery of viruses from the drinking water supply of a major city underscores the importance of the development of such methods.

A SENSITIVE QUANTITATIVE METHOD FOR DETECTING SMALL QUANTITIES OF VIRUS IN LARGE VOLUMES OF WATER.

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Development of a method for detecting small quantities of virus in large quantities of water is important because it is conceivable that water may serve as an important vehicle by which small amounts of viruses enter susceptible communities after which the viruses may be widely transmitted by personal contact. Low level water-borne infections are hard to detect by classical epidemiological methods because such infections, even if they always produced clinical disease, would be difficult to distinguish from those transmitted by personal contact.

A sensitive epidemiological method for detecting low level water-borne virus transmission has been described (1), but has not yet been tested. However, recent disclosure that viruses were detected by a newly devised qualitative technic in almost 19 percent of 210 water samples taken in 1962 from various points in the distribution system of Paris, France (2) lends immediate import to the development of a sensitive quantitative method for detecting small quantities of virus in large quantities of water.

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Initial efforts to detect small amounts of viruses in large volumes of water were made by adsorbing virus onto monkey kidney cells in suspension. Fifty to 100 plaque forming units (PFU) of poliovirus 1 were added to a 200 ml suspension of versenated primary African Green monkey (Cercopithecus aethiops sabaeus) kidney cells and the two were agitated by a magnetic stirrer for 1 hour. The suspending menstruum was Earle's balanced salt solution (BSS), 99.5 percent; lactalbumin hydrolysate, 0.5 percent; and penicillin, streptomycin, and nystatin, 50 μg each/ml (maintenance medium). Cells were then packed by low speed centrifugation, resuspended in 200 ml of growth medium (Hanks' BSS, 96.5 percent; lactalbumin hydrolysate, 0.5 percent; calf serum [free of poliovirus 1 antibody], 3 percent; penicillin, streptomycin, and nystatin, 50 μg each/ml), and 2 ml per tube were planted in about 100 roller tubes. Sufficient cells were used so that confluent cultures formed on the glass within 24 hours at 36.5°C. After attaching to the glass, these cultures were incubated in roller drums and microscopically examined periodically for one week for cytopathic change (CPC) produced by the virus. Over 40 percent recovery of virus was achieved, and it appeared that a system might be constructed in which large volumes of virus-laden fluids would pass sufficiently close to susceptible cells to be adsorbed in large percentage.

A glass tube, about 2.5 cm in diameter and about 30 cm long, was placed vertically on a stand. Fifty to 100 PFU of poliovirus 1 suspended in maintenance medium were introduced into the tube from above at the rate of 1 liter per hour and passed through 5 to 10 cc of packed versenated first-passage kidney cells that rested on a Fiberglas mat. Recovery of only 1 or 2 percent of the virus suggested that the time of proximity between the virus and susceptible cells needed for

quantitative recovery of the virus in this system was greater than could probably be achieved.

To determine the time of proximity between cells and virus required for quantitative recovery of virus, groups of 15 cell cultures in roller tubes were drained of fluid and each culture was inoculated with 2 ml of maintenance medium containing about 1.5 PFU of poliovirus 1. At various intervals, the fluid in each tube of a group was removed and replaced with 2 ml of maintenance medium containing no virus. Periodically, during the 10 days after inoculation, the cultures were examined microscopically. Only 7 to 20 percent of the tubes in which the medium was replaced 3 to 5 hours after inoculation contained virus. Even when fluids were not changed until 48 hours after inoculation, only 67 percent contained virus.

It now appeared that quantitative recovery of virus by adsorption to susceptible cells could be achieved only by permitting extended contact between the virus-laden fluid and the cells. We wanted to test large quantities of fluid, but it seemed unlikely that the virus would survive long enough to permit quantitative recovery, if in fact it could negotiate the long distance to the cells at all. However, since this now appeared to be the most promising direction with this system, experiments were performed to determine whether virus could be adsorbed quantitatively by cells from larger volumes of fluid during extended periods of proximity.

Each of 10 cultures of African Green monkey kidney cells grown in 6-oz prescription bottles, was washed three times with maintenance medium and inoculated with 170 ml of this same medium containing approximately 5.49 PFU of poliovirus 1 (Test A). Dilutions of 1:2 (Test B)

and 1:10 (Test C) were made from the preparation used in Test A, and in each test a 170-ml volume was inoculated onto each of 10 cell cultures. Additionally, each of 20 cultures was inoculated with 85 ml of Test B suspension (Test B1), and each of 38 cultures was inoculated with 40 ml of Test B suspension (Test B2). Cultures were examined microscopically for CPC every several days for 14 days. The number of PFU in the test suspensions was determined by assaying in 7 cell cultures the virus suspension from which the dilution for Test A was prepared. In this plaque assay, virus adsorbed to cells for 2 hours at room temperature (24° to 26°C) before the cells were overlayed. The technics for the preparation of cell cultures and plaque assays have been described (3) and so have been the methods for determining the most probable number (MPN) values (4, 5). All cell cultures propagated in prescription bottles had an area of 4,500 mm^2.

The results of Test B2 (Table 1) demonstrated quantitatively the relative sensitivity of this method for recovering small amounts of virus from relatively large volumes of fluid. If a maximum error of about 35 percent is assumed in the precision of the plaque assay (6), recovery of a quantity of virus at least equal to the PFU inoculated appears probable. The narrow 95 percent confidence limits obtained with 38 cell cultures lends considerable weight to this conclusion.

In Tests A, B, and C, with 170-ml volumes, the 95 percent confidence limits were very wide because only 10 replicates were used in each test. The consistent pattern of higher PFU:MPN ratios in Tests A, B, and C as compared to those in Tests B1 and B2 suggested lower recovery efficiencies with larger volumes of fluid (increased depth over the cells). This impression was strengthened by a similar

pattern of increasing recovery efficiencies with decreasing fluid volumes in Tests B, B1, and B2 in which different quantities of the same virus suspension were tested.

Because of the wide spread of confidence limits in most of these tests, another experiment was undertaken to confirm the results in Table 1. A series of dilutions of poliovirus 1 was prepared containing 0.235 PFU per 50, 100, and 150 ml as determined by plaque titration in 10 cell cultures of the virus pool from which the dilutions were made. Quantities of 50, 100, and 150 ml were inoculated onto each of a corresponding group of 40 cell cultures, each of which had been first washed three times with maintenance medium (Table 2, D, E, and F). These cultures were incubated at 35°C, and examined microscopically for CPC periodically for 14 days. A second experiment, similar to the first, was prepared with a slightly larger virus concentration (Table 2, G, H, and I). A third experiment, also similar to the first, was undertaken with changes in volumes and virus concentrations (Table 2, J, K, L, and M).

Tests D and E, and G and H, respectively, demonstrate that virus recovery decreased in proportion to volume increase when volumes were 100 ml or less in 6-oz prescription bottles. Increases in volume beyond 100 ml per bottle culture produced equivocal results (Tests F and I).

Tests K, L, and M suggest, with volumes of 50 ml and less, there was not a proportional decrease in virus recovery when volume was increased. A four-fold increase in volume reduced sensitivity only 50 percent. This implies that to a limit (perhaps between 50 and 100 ml), the sensitivity of the system under field conditions may be increased by testing larger volumes per bottle culture.

Further testing will determine optimal volume (depth) levels that will yield maximum sensitivity. Fluid volume on the cell cultures reflects fluid depth, and fluid depth is probably the determinant of the efficiency of virus recovery. Nonetheless, the data demonstrate both the high sensitivity and the quantitative nature of the system.

The inoculum in Test J is 25% greater than that in Test K. When adjustment is made for this difference, the sensitivity of the 0.5-ml inoculum is not significantly greater than the sensitivity of the 12-ml inoculum.

Absolute sensitivity cannot be measured at present since the plaque count is not an absolute value but an alterable one that is influenced by variations in the plaquing procedure. However, the plaque count is a valid marker against which the relative efficiency of a recovery procedure can be measured quantitatively.

The microscopic examinations of the cell cultures presented an unexpected problem. The CPC produced by the virus in bottle cultures was different from the CPC produced by this same virus in roller tube cultures. At 40X magnification, the CPC in prescription bottles was characterized by rounded cells, somewhat smaller than those in roller tube cultures. The same degree of refractility and characteristic grotesqueness of the affected cells that occurred in roller tubes did not occur in bottle cultures. Large areas of retraction and detachment appeared also in bottle cultures. For confirmation, fluid from each positive culture in the test was passed to a roller tube where characteristic CPC appeared. Fluids from all apparently negative cultures were passed also. Three of these, two in Test H1 and 1 in Test C, contained virus. In subsequent tests, this experience was repeated occasionally,

though cultures seldom became positive after more than 8 days following inoculation when incubated at 35°C.

Cell sheets remained in acceptable condition for the 14-day period of the tests, though no fluid changes were made. Cultures with small volumes of fluid often held up better than cultures with large volumes of fluid. The pH of the maintenance medium was 7.4 at the beginning of the tests and about 6.8 at the end of the tests.

Maximum sensitivity of the cell culture system probably depends upon several conditions. To determine the sensitivity of the system, we undertook a preliminary study with minimal fluid volume, and also with low preliminary incubation temperatures and higher fluid volumes.

A series of dilutions of poliovirus 1 were prepared containing 0.25, 0.5, or 1.0 PFU per 0.5 ml as determined by plaque titration in 10 cell cultures of the virus pool from which the dilutions were made. Half-ml amounts of each dilution were inoculated into each of a corresponding group of cell cultures (Table 3, N1, O1, or P1). After an adsorption period at room temperature (24° to 26°C), 50 ml of maintenance medium was introduced onto each cell culture and the cultures were incubated at 35°C for 14 days more.

The dilutions containing 0.25, 0.5, and 1.0 PFU of poliovirus 1 per 0.5 ml of medium were each diluted 1:100, and 50-ml quantities of the diluted fluids containing poliovirus 1 concentrations of 0.25, 0.5, or 1.0 PFU per 50 ml, respectively, were inoculated onto each of a corresponding group of bottle-cell cultures (Table 3, N, O, and P). Most cultures were incubated for 2 or 5 days at room or refrigerator temperatures, then at 35°C for 14 days or more.

From the beginning of the test, control cultures were incubated at 35°C for 14 days.

Table 3 demonstrates no significant difference in recovery efficiency among cultures pre-incubated at low temperatures as compared with those incubated only at 35°C. Tests in which cultures inoculated with only 0.5 ml of fluid were incubated for 2, 4, or 24 hours at 24° to 26°C before 50 ml of maintenance medium was added to each culture yielded results similar to those in which 50 ml volumes were inoculated initially. Because of the wide confidence limits, however, further testing is indicated.

The data indicate that low incubation temperature does not facilitate adsorption of virus to cells. We have been aware for some time that adsorption of poliovirus 1 and other enteroviruses is as efficient at 24° to 26°C as at 36°C (3). Low pre-incubation temperatures may be useful, however, with viruses of lower thermostability.

Characteristically, delayed CPC occurred in cultures pre-incubated at low temperatures. The virus probably adsorbed to cells at low temperatures (3), but higher temperatures were required for the complete reproductive cycle of the virus to take place. When low-temperature pre-incubation preceded incubation at 35°C, CPC continued to occur regularly more than 8 days after inoculation of the virus.

Since the methodology under development is directed at eventually testing large quantities of potable waters for virus, a test was prepared to determine whether such water is toxic to African Green monkey kidney cell cultures. Maintenance medium, prepared with tap water, produced no apparent toxicity in cell cultures after incubation for 14 days at 35°C with no medium changes.

Our efforts are now directed towards increasing the sensitivity of the detection procedure, and at increasing the volumes of water that, practically, can be tested for virus. We intend also to study the usefulness of continuous high speed centrifugation, reverse osmosis, evaporation, flotation and other concentration methods in lieu of, and in conjunction with, the procedure we have described.

The applicability of the method described for isolating small quantities of virus from large volumes of liquids other than water, or from solid materials is apparent.

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Table 1. Effect of fluid volume (depth) on recovery of poliovirus 1 on monkey kidney epithelial cell cultures.

Test	Fluid volume on cell culture (ml)	Depth of fluid over cells (mm)	PFU seeded onto each cell culture	No. of cell cultures inoculated	No. of cell cultures not infected	Most probable number (MPN)/ cell culture	95% confidence limits
A	170	27.9	5.49	10	1	2.30	0.92-5.30
B*	170	27.9	2.75	10	3	1.20	0.48-2.44
C+	170	27.9	0.55	10	7	0.35	0.09-0.96
B1‡	85	15.6	1.37	20	2	2.30	1.21-4.02
B2‡	40	8.8	0.65	38	9	1.44	1.30-1.58x

*A 1:2 dilution of the preparation used in Test A.

+A 1:10 dilution of the preparation used in Test A.

‡Same virus suspension as that used in Test B.

xComputed from the standard error.

Table 2. Effect of fluid volume (depth) on recovery of poliovirus 1 on monkey kidney epithelial cell cultures.

Experiment	Test	Fluid volume on cell culture (ml)	Depth of fluid over cells (mm)	PFU seeded onto each cell culture	No. of cell cultures inoculated	No. of cell cultures not infected	Most probable number (MPN)/ cell culture	95% confidence limits*
1	D	50	10.6	0.24	40	30	0.29	0.15-0.43
	E	100	18.1	0.24	39	33	0.17	0.05-0.29
	F	150	25.5	0.24	40	38	0.05	< 0.01-0.12
2	G	50	10.6	0.3	40	26	0.43	0.28-0.58
	H	100	18.1	0.3	40	33	0.19	0.07-0.31
	I	150	25.5	0.3	40	31	0.25	0.12-0.38
3	J	0.5**	-	0.40	40	9	1.49	1.36-1.62
	K	12	3.8	0.32	40	16	0.92	0.77-1.07
	L	25	6.2	0.33	39	18	0.77	0.61-0.93
	M	50	10.6	0.33	40	25	0.47	0.32-0.62

*Computed from the standard error.
**Cell cultures were degenerating for lack of medium within 2 days after virus inoculation. Five ml of maintenance medium was added to each culture 9 days after virus inoculation, after which medium from each bottle culture was passed to roller tube cultures.

Table 3. Effect of fluid volume (depth) and low temperature preincubation on quantitative recovery of poliovirus 1 from fluid.

Test	Fluid volume on cell culture (ml)	Initial depth of fluid over cells (mm)	Preliminary* incubation Temp. (°C)	Preliminary* incubation Time**	Volume of media added after preliminary incubation (ml)	PFU seeded onto each cell culture	No. of cell cultures inoculated	No. of cell cultures not infected	Most probable number (MPN)/ cell culture	95% confidence limits
N	50	10.6	24-26	2d	None	1.0	10	3	1.20	0.48-2.44
				5d	None	1.0	10	2	1.61	0.51-3.30
			4	2d	None	1.0	10	4	0.92	0.31-1.90
				5d	None	1.0	10	2	1.61	0.51-3.30
			-	None	None	1.0	20	5	1.39	0.76-2.26
N1	0.5	-	24-26	4h	50	1.0	20	5	1.39	0.76-2.26
O	50	10.6	24-26	2d	None	0.5	10	9	0.11	0.01-0.51
				5d	None	0.5	10	6	0.51	0.16-1.32
			4	2d	None	0.5	10	7	0.36	0.09-0.96
				5d	None	0.5	10	5	0.69	0.25-1.51
			-	None	None	0.5	19	12	0.46	0.16-1.01
O1	0.5	-	24-26	2h	50	0.5	20	10	0.69	0.35-1.23
				4h	50	0.5	20	8	0.91	0.43-1.57
				24h	50	0.5	20	9	0.79	0.35-1.51
P	50	10.6	24-26	2d	None	0.25	10	4	0.92	0.31-1.90
				5d	None	0.25	10	8	0.22	0.04-0.92
			4	2d	None	0.25	10	4	0.92	0.31-1.90
				5d	None	0.25	10	6	0.51	0.16-1.32
			-	None	None	0.25	20	14	0.36	0.15-0.76
P1	0.5	-	24-26	4h	50	0.25	20	13	0.43	0.15-0.89

*Subsequent incubation was at 35°C for 14 days.
**d = days, h = hours.